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Intermittent Selection Pressure With Zidovudine Plus Zalcitabine Treatment Reduces the Emergence In Vivo of Zidovudine Resistance HIV Mutations

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The development of mutations conferring drug resistance was investigated in 49 antiretroviral-naive asymptomatic HIV-1 subjects with $CD4^+$ cell counts of 250–500/mm^3 given intermittent (6-week courses, 6 weeks apart) or continuous treatment with zidovudine (AZT) plus zalcitabine (ddC) over 54 weeks. The concentration of human immunodeficiency virus type 1 RNA in the plasma and the CD4 cell counts were measured every 6 weeks. The rate of decrease of HIV-1 RNA concentration in plasma after a 6-week course of AZT + ddC was similar for each treatment cycle (approximately 1-log reduction). The plasma HIV-1 RNA concentration returned to its initial level at each treatment interruption. The mean CD4 cell counts after 54 weeks in the two treatment groups were similar. Genotype analysis by sequencing the reverse transcriptase coding region from plasma viral RNA on treatment showed a lower frequency of AZT resistance mutations after 54 weeks in patients given intermittent treatment (18%) than in those treated continuously (79 %, $P < 0.001$). No mutations conferring ddC resistance or multidideoxynucleoside resistance were observed in either group. These findings may have clinical implications for long-term treatment strategies. ***J. Med. Virol. 57:163–168, 1999.***

KEY WORDS: zidovudine; zalcitabine; resistance mutations; intermittent treatment

INTRODUCTION

Virus replication is typically highly dynamic in human immunodeficiency virus (HIV) infections [Ho et al., 1995; Wei et al., 1995]. The errors of the reverse transcriptase (RT) (3.4×10^{-5} misincorporations per replication cycle in vivo) [Mansky and Temin, 1995], the recombination events during the viral replication, and the high rate of virus production (up to 10^{10} virions per day) [Coffin, 1995] result in multiple variants within an infected individual. There is also rapid selection for adapted viruses via coreceptor selection, immune pressure, and/or effective antiviral drugs [Wei et al., 1995]. Potent inhibitors of viral replication may reduce the amount of plasma HIV-1 RNA to levels that are undetectable even by sensitive assays. Increases in plasma RNA levels despite continuing therapy are often due to the replication of drug-resistant mutant viruses selected from the virus population by antiretroviral treatment [Ho et al., 1995; Loveday et al., 1995; Schuurman et al., 1995; Wei et al., 1995]. Mutations giving resistance to zidovudine (AZT) have been identified at codons 41, 67, 70, 215, and 219 of the HIV RT gene [Larder and Kemp, 1989; Kellam et al., 1992]. The genotypes most frequently associated with high-level AZT resistance and clinical failure are combinations of mutations at positions 41 and 215 or 67, 70, and 215 [Boucher et al., 1992]. The clinical benefit of combination therapy with AZT plus zalcitabine (ddC), or AZT plus didanosine (ddI), has been demonstrated for antiretroviral-naive, HIV-1–infected individuals; they slow the progression to symptomatic disease and prolong survival [Delta Coordinating Committee, 1996; Hammer et al., 1996; Saravolatz et al., 1996]. However, several studies have shown that the emergence of AZT resistance mutations was not prevented by these combination treatments [Richman et al., 1994; Schafer et al., 1995; Larder et al., 1996; Schooley et al., 1996; Brun-Vézinet et al., 1997], thus limiting the options for future treatment and effective long-term therapy.

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Little is known about the effect of mutations conferring resistance to AZT on viral fitness. In vitro studies have shown that drug-resistant mutants have a lower replication capacity than the wild-type virus in the absence of drug selection pressure [Boucher et al., 1993; Chow et al., 1993]. The lower replication capacity of drug-resistant mutants may also account for the low frequency of drug-resistant mutants in virus populations from untreated individuals [Najera et al., 1994, 1995]. Thus, interruptions in the selection pressure imposed by AZT plus ddC treatment may delay the emergence in vivo of AZT-resistance mutations because the mutant viruses have a lower replication capacity than wild-type viruses in the absence of drugs.

This 54-week study of antiretroviral-naive asymptomatic HIV-1–infected subjects with $CD4^+$ cell counts of 250–500/mm^3 was designed to assess whether intermittent treatment with AZT plus ddC (6-week courses, 6 weeks apart) resulted in fewer AZT-resistance mutations than the same treatment given continuously. Genotypes were analyzed before treatment and after 54 weeks of treatment by sequencing of the RT-coding region of viral RNA found in plasma samples. The HIV-1 RNA concentration in the plasma and CD4 cell counts were also determined every 6 weeks.

MATERIALS AND METHODS

Patients

Antiretroviral-naive asymptomatic HIV-1–infected individuals with CD4 cell counts of 250–500/mm^3 were randomly assigned to one of two groups. One group was given an intermittent regimen (6-week courses, 6 weeks apart) of AZT (250 mg twice daily) plus ddC (0.75 mg three times daily) (group I), and the other a continuous regimen of AZT (250 mg twice daily) plus ddC (0.75 mg three times daily) (group II). Informed consent was obtained from all participating subjects. Blood samples were collected 6 weeks before and immediately before treatment began, then every 6 weeks throughout the study. Plasma samples were prepared within 3 hr of blood collection, aliquoted and stored at −80°C. PBMCs were isolated from blood samples by the Ficoll-Hypaque density method. Mononuclear cells were counted, divided into several aliquots, each with 1×10^6 cells, and stored as pellets at −80°C.

HIV-1 RNA in Plasma

The HIV-1 RNA in plasma samples was measured using the Amplicor HIV-1 Monitor reverse transcriptase–PCR assay (Roche Diagnostic Systems, Neuilly, France) according to the manufacturer's instructions. The cutoff value for this assay was 200 copies/ml.

Cell-Associated HIV-1 RNA

Intracellular HIV-1 RNA levels in PBMC were determined by PCR, with a modified Amplicor HIV-1 Monitor RT-PCR assay, after elimination of extracellular RNA [Tamalet et al., 1997]. Cells were treated with DNase (Boehringer-Mannheim, Mannheim, Germany) before amplification to ensure that only viral RNA was detected. The detection limit was 50 copies per 10^6cells.

Genotype Analysis of HIV-1 RT

Both strands of a nested PCR product from the HIV-1 genome encoding the first 240 amino acids of the RT were sequenced directly. The outer primers were RT2 (antisense primer, 5'-TCTACTTGTCCATGCATGGCTTC-3') and RT1 (sense primer, 5'-GGAAACCAAAAATGATAGGGGGAATTGGAGG-3'); the inner primers were RT4 (antisense primer, 5'-ATGTCATTGACAGTCCAGCT-3') and RT3 (sense primer, 5'-ATTTTCCCATTAGTCCTATT-3'). RNA was extracted from 100 μl serum by the guanidinium thiocyanate-phenol-chloroform method. Reverse transcription was carried out with 20 units M-MuLV reverse transcriptase (Boehringer-Mannheim) and the primary antisense primer. PCR was carried out in 10-mM Tris-HCl (pH 8.3), 50-mM KCl, 1.5-mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 50 pmol of each primer, 2.5-units *Taq* polymerase (AmpliTaq, Perkin Elmer Cetus, Norwalk, CT), and 5-μl cDNA solution. The primary PCR involved initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 60 sec; annealing at 55°C for 60 sec, and polymerization at 72°C for 150 sec with a final elongation at 72°C for 10 min. An aliquot (5 μl) of the primary PCR products was used for 35 cycles of nested PCR as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and polymerization at 72°C for 60 sec, with a final elongation at 72°C for 5 min. The RT3-RT4 amplification gave an 800-bp product, which was purified using QIAamp columns (Qiagen, Courtaboeuf, France) and sequenced on both strands by the dideoxy chain termination method (ABI PRISM Ready Reaction AmpliTaq Fs, Dye Deoxy Terminators, Applied Biosystems, Paris, France) on an ABI377 automated DNA sequencer (Applied Biosystems). All the measures to prevent contamination suggested by Kwok and Higushi [1989] were strictly applied. Mutational analysis was done after sequence alignment with the CLUSTALW program [Thompson et al., 1996]. Nucleotide heterogeneity was compared for nucleotide sequences at critical codons associated with AZT resistance [Larder and Kemp, 1989] and ddC resistance [Fitzgibbon et al., 1992; Gu et al., 1994; Zhang et al., 1994]. Mutations conferring resistance to other nucleoside analogues [Schinazi et al., 1996] and multidideoxynucleoside resistance mutations [Shafer et al., 1994; Shirasaka et al., 1995; Schmit et al., 1996] were also examined.

CD4 Lymphocyte Counts

Peripheral blood CD4 lymphocytes were counted by flow cytometry (Epics Profile; Coulter, Hialeah, FL) using commercially available monoclonal antibodies (Beckton Dickinson, Mountain View, CA).

Statistical Analysis

HIV-1 RNA concentrations were transformed to $\log_{10}$ values before analysis. Specimens in which HIV-1 RNA

TABLE I. Baseline Characteristics of 49 Antiretroviral-Naive Asymptomatic Subjects Given Either Intermittent Zidovudine Plus Zalcitabine Therapy (6-Week Courses, 6 Weeks Apart) (Group I) or Continuous Zidovudine Plus Zalcitabine Therapy (Group II)

	Group I	Group II
Number of patients	27	22
Mean age (years)	38	35
Sex (male/female)	22/5	17/5
CD4 cell count (cells/mm^3), mean ± SD	383 ± 88	358 ± 96
Plasma HIV-1 RNA (copies/ml), mean ± SD	4.34 ± 0.75	4.20 ± 0.66

was undetectable were assigned the value of the detection limit of the Monitor assay (i.e., 2.3 log for RNA in plasma and 1.7 log for RNA in PBMC). Correlations between variables were calculated by Spearman's rank correlation. Quantitative variables were analyzed by Mann-Whitney test. Qualitative variables were analyzed by Chi-square test or Fisher's exact probability test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Patient Characteristics Before Treatment

Fifty-four antiretroviral-naive asymptomatic HIV-1–infected individuals were randomized between May and December 1995. Five patients were lost to follow-up and were excluded from the analysis. Table I shows the baseline clinical features and laboratory tests for the 27 patients who were given intermittent AZT + ddC therapy (group I) and the 22 patients who were given continuous AZT + ddC therapy (group II). There were no significant differences in the baseline characteristics of the two groups.

Changes in HIV-1 RNA Concentration and CD4 Cell Count

Sixteen of the 27 patients in group I completed the 54 weeks of intermittent AZT + ddC therapy and 11 discontinued treatment because of intolerance (n = 2), a decrease in CD4 cell count to <200/mm^3 (n = 3), or for personal reasons (n = 6). Seventeen of the 22 patients in group II completed the full 54 weeks of AZT + ddC therapy and 5 discontinued treatment because of intolerance (n = 2), a decrease in CD4 cell count to <200/mm^3 (n = 1), or for personal reasons (n = 2).

Figure 1 shows the changes in plasma HIV-1 RNA concentration over time. HIV-1 RNA was consistently low in group II patients throughout the study period (−1 log after 6 weeks to −0.9 log after 54 weeks). In group I, the rate of decrease in HIV-1 RNA in plasma after a 6-week course of AZT + ddC was similar for each cycle of treatment (approximately −1 log). The plasma HIV-1 RNA returned to its initial level each time treatment was interrupted. Intracellular HIV-1 RNA was assayed for 10 patients from group I at baseline, week 6, and week 12. Changes in intracellular HIV-1 RNA levels were correlated with the changes in plasma HIV-1 RNA (data not shown). The changes in CD4 cell count over time are shown in Figure 2. Mean CD4 cell count was lower in group I patients after the 6-week interruption of each treatment cycle. However, the mean CD4 cell counts after 54 weeks in groups I (433 ± 134 cells/mm^3) and II (456 ± 148 cells/mm^3) were similar.

Emergence of Viral Mutations Conferring Resistance

The RT gene was sequenced from codon 1 to 240 for paired plasma samples taken at the start of the study and at week 54 or at the end of treatment for patients who discontinued their treatment. The presence of resistance mutations was not assessed at the start of the study for three plasma samples with <1,500 HIV-1 RNA copies/ml (1 in group I, 2 in group II) and at the end of follow-up for 13 plasma samples in which the amount of HIV-1 RNA was below the detection limit of the Monitor assay (5 in group I, 8 in group II). At the start of the study, one patient from group I had a virus with AZT resistance mutations, D67N-K219Q. The remaining 45 evaluable patients had wild-type viruses. At the end of follow-up, mutations associated with AZT resistance were detected in 4 patients from group I (1 D67N-K70R-K219Q, 1 M41L-T215Y, 1 K70R, and 1 T215Y), of whom 1 had AZT resistance mutations at the start of the study, and in 11 patients from group II (4 M41L-T215Y, 4 T215Y, 1 M41L-D67N-T215Y, 1 M41L-K70R, and 1 D67N-K70R). No mutations associated with ddC resistance were observed in either group. No multidideoxynucleoside resistance mutations were detected. Thus, the incidence of mutations conferring resistance to AZT was significantly lower in patients given intermittent AZT + ddC treatment (4 /22, 18%) than in patients given continuous AZT + ddC treatment (11/14, 79%) ($P < 0.001$).

DISCUSSION

This study shows that intermittent AZT plus ddC therapy results in the slower emergence in vivo of AZT resistance than does continuous therapy. In vitro studies have shown that drug-resistant mutants have lower replication capacity than wild-type virus in the absence of drug selection pressure [Boucher et al., 1993; Chow et al., 1993]. It has also been reported in a subject newly infected with an AZT-resistant HIV-1 strain that the viral population with tyrosine at codon 215 of RT, which confers resistance, was gradually replaced in the absence of drug [Goudsmit et al., 1997]. Thus, the lower incidence of AZT resistance in patients given intermittent treatment may be due to HIV variants with RT drug-resistance mutations being less fit than the wild-type virus. Resistant viruses are probably selected after 6 weeks of therapy but are not detected because population sequencing methods cannot detect minor viral populations (approximately 20% sensitivity for a given variant). When the drugs were withdrawn, the original selection pressure returned, favoring the wild-type virus.

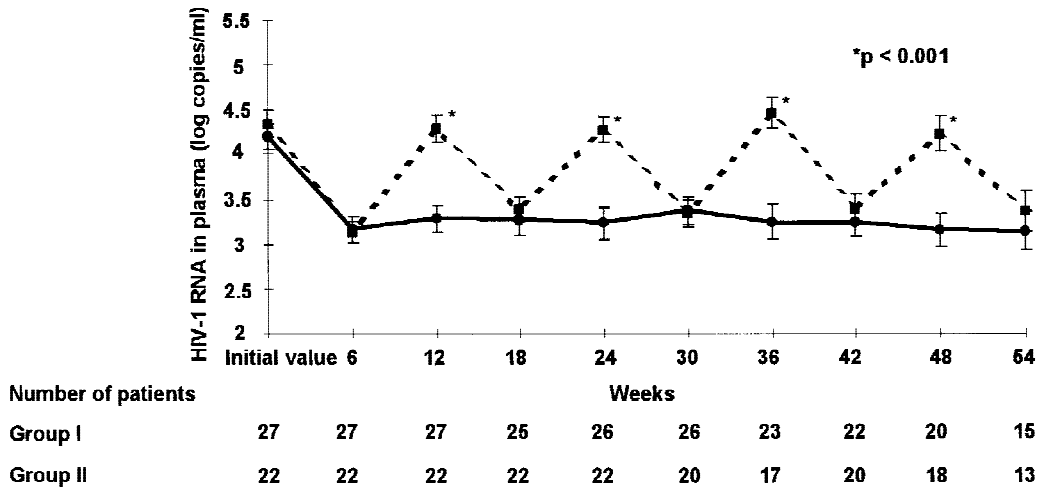

Fig. 1. Changes in HIV-1 RNA concentration in plasma (means ± SE) for patients given intermittent (group I) or continuous (group II) zidovudine plus zalcitabine treatment. *P* values are for differences between patients given intermittent (■) and continuous (●) treatment.

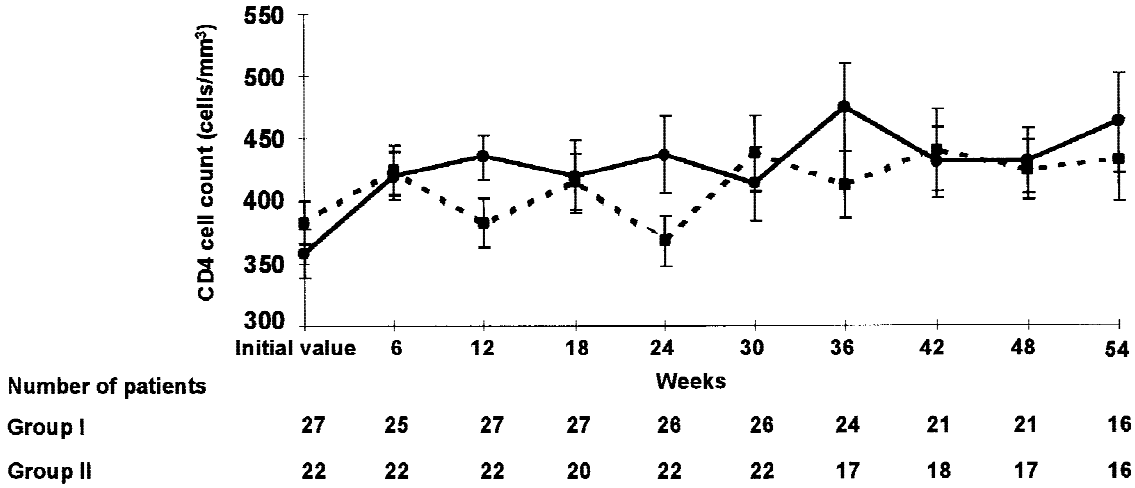

Fig. 2. Changes in CD4 cell counts (means ± SE) for patients given intermittent (group I) or continuous (group II) zidovudine plus zalcitabine treatment. Differences between patients given intermittent (■) and continuous (●) treatment were not significant.

This study was designed before protease inhibitors came into general use. The best strategy for preventing the development of drug resistance is the complete suppression of viral replication by highly active antiretroviral therapy (HAART) regimens involving two nucleoside analogues and a protease inhibitor [BHIVA et al., 1997; Carpenter et al., 1997]. However, there is evidence of residual replication in many patients when plasma HIV-1 RNA is assayed with the most sensitive assays available (detection limit 20 to 50 copies/ml). This might eventually lead to the emergence of drug resistance, limiting the options for future treatment and effective long-term therapy. Replication-competent virus can also be recovered from resting $CD4^+$ T-lymphocytes in patients successfully treated with HAART [Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997], suggesting that the treatment time required to eradicate HIV-1 from an infected person may be longer than that estimated from plasma virus decay characteristics [Perelson et al., 1997]. The success of protease inhibitors must also be tempered by their potential long-term toxicity [Lo et al., 1998; Miller et al., 1998]. Thus, alternative strategies, such as a reduction in drug selection pressure, may facilitate long-term control of viral replication with less toxicity for the patient. Indeed, most HIV variants with resistance to protease inhibitors have a reduced replicative capacity due to an impairment of protease function [Croteau et al., 1997; Zennou et al., 1998]. But in some cases, partially compensatory mutations in Gag cleavage sites have been described.

A major problem of treatment interruption is that the plasma HIV-1 RNA returns to its initial level because virus production recovers rapidly. Similar results were recently reported for patients given protease inhibitor therapy with treatment interruptions [Neumann et al., 1998]. The changes in CD4 cell count relative to the changes in plasma and cell-associated HIV-1

RNA levels in patients given intermittent treatment provides further evidence that CD4 cells are destroyed as a result of HIV replication. Nevertheless, the increase in CD4 cell counts after 54 weeks for patients given intermittent and continuous treatments was similar. The three patients given intermittent treatment whose CD4 cell counts decreased also had high levels of HIV-1 RNA in their plasma at the start of the study (>5.3 log). Therefore, patients with low plasma HIV-1 RNA concentrations, less than 4 log in the absence of drug, may experience less cell destruction at each treatment interruption.

An intermittent antiretroviral regimen may therefore delay the emergence of mutations conferring resistance to antiviral drugs. Treatment interruptions may accelerate CD4 cell destruction, so patients with low initial plasma HIV-1 RNA concentrations would probably derive the most benefit from intermittent selection pressure. If viral eradication is considered to be hypothetical event requiring prolonged complete suppression of viral replication, then intermittent antiviral regimens may buy time in which stronger and more tolerable regimens can be developed. It must be underlined that current recommendations for antiretroviral treatment of HIV seropositive individuals are continuous regimens combining reverse transcriptase and protease inhibitors. Nevertheless, the clinical implications of our results, particularly for long-term treatment strategies, should be investigated further.

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